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PRINCIPAL INVESTIGATOR: Todd W. Miller, Ph.D.

CONTRACTING ORGANIZATION: New York State University
at Stony Brook
Stony Brook, New York 11794-3366

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This project focuses on the product of the Her2/Neu oncogene, a receptor tyrosine kinase (Neu) that is amplified in 25-30% of human primary breast tumors. The goal of this project is to characterize the substrate specificity of Neu using peptide libraries. This report covers the second year of the project. We have isolated Neu from Sf9 cells using a baculovirus expression vector. We have isolated five novel sequences from peptide libraries that are phosphorylated by Neu, and determined the kinetic parameters for phosphorylation using individually-synthesized peptides. One of the sequences, AAEEIYAARRG, is the best synthetic peptide substrate reported to date for Neu. We synthesized potential peptide-based inhibitors of Neu by replacing the tyrosine in this peptide and another sequence with p-carboxy-Phe. The inhibitory potency of this first generation of inhibitors toward Neu was somewhat low. Thus, we plan to test additional inhibitors before proceeding to the final stage of the project, namely examining in vivo inhibition of Neu.

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W Todd Miller 6/15/99

PI - Signature Date

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INTRODUCTION

Constitutive activation of signaling pathways involving tyrosine phosphorylation is believed to play a crucial role in human breast cancer. The receptor tyrosine kinase $p185^{HER2/neu}$ (Neu) is amplified in 20 to 30% of human primary breast cancers, and expression of Neu in tumor tissues is correlated with poor clinical prognosis (1). Transfection of NIH3T3 cells with the *neu* oncogene results in malignant transformation (2), and studies in transgenic mice that overexpress Neu indicate that breast tissue is particularly susceptible to the transforming properties of the *neu* oncogene (3). Neu is a 185 kilodalton protein that is structurally very similar to the EGF receptor: it has a cysteine-rich extracellular domain, a membrane-spanning region, and an intracellular tyrosine kinase domain (4). The mechanism by which Neu transmits its mitogenic signal is not completely clear, but the tyrosine kinase activity of the receptor plays a crucial role (1). The identities of the *in vivo* substrates for Neu are unknown, and the substrate specificity of the Neu tyrosine kinase has not previously been defined. The broad goal of this project is to obtain information about the substrate specificity of the Neu tyrosine kinase. This information will provide a clearer understanding of how this enzyme subverts the normal signalling pathways in cells to cause neoplastic growth. In the second phase of the project, we will attempt to design molecules that specifically interfere with the action of Neu tyrosine kinase *in vivo* and *in vitro*. The results of these investigations may suggest avenues for design of specific anticancer agents.

The specific objectives of our project are:

Objective 1. We will isolate the Neu tyrosine kinase from cultured breast cancer cells and study its specificity using combinatorial peptide libraries.

Objective 2. We will also study the specificity of Neu with a solid-phase tyrosine kinase assay.

Objective 3. Nonphosphorylatable tyrosine mimetics will be introduced into peptide sequences obtained from Objectives 1 and 2. These compounds will be tested as inhibitors of the Neu tyrosine kinase *in vitro*. Results will be compared for representatives of other classes of tyrosine kinases.

Objective 4. Active compounds from Objective 3 will be truncated stepwise from the N- and C-termini to determine the minimum peptide length necessary for specific inhibition of Neu. The resulting peptides will be tested as inhibitors of the proliferation of a breast cancer cell line, SKBR3, using a tritiated thymidine incorporation assay.

BODY OF REPORT

We have completed the studies proposed for Technical Objective 1 (Tasks 1-4) and Technical Objective 2 (Tasks 1-4). Work is in progress on Technical Objective 3 (Tasks 1 and 2).

Objective 1. Studying Neu specificity with peptide libraries.

Task 1. Purification of Neu. In the June 1998 progress report, we reported that the production of Neu in large amounts presented a problem. We overcame this experimental hurdle in the past year by the following approaches:

(i) we subcloned the DNA encoding the tyrosine kinase catalytic domain of Neu into the *E. coli* expression vector pGEX-2T. This allowed us to express this domain of Neu as a fusion protein with glutathione S-transferase in bacteria. This strategy produced a stable protein of the expected molecular weight which we were able to purify to homogeneity (Fig. 1). Unfortunately, the tyrosine kinase activity of this isolated tyrosine kinase domain was very low (data not shown). We concluded that this kinase would not be active enough for our subsequent experiments.

(ii) We prepared full-length Neu by infecting Sf9 insect cells with baculovirus encoding the V664E Neu (t-Neu) receptor, which was kindly provided by Mark I. Greene from University of Pennsylvania. The baculovirus was added to 600mL of cells at a multiplicity of infection of 1:20, and cells were harvested at three days post-infection. t-Neu receptor was partially purified by anion chromatography using HiLoad Q Sepharose (Figure 2). The Neu kinase we expressed is composed of 1260 amino acid residues, and it migrates with an apparent molecular weight of 185 kilodaltons on 8% SDS-PAGE. Protein expression in Sf9 cells peaked at 3 days post-infection as determined by Western blotting with anti-phosphotyrosine antibody. Sf9 insect cells were harvested by centrifugation at 5,000g. The cell pellet was resuspended in 20mL of lysis buffer (50mM Tris pH 8.0, 2mM EGTA/EDTA, 0.1% β-mercaptoethanol, 10% glycerol, 0.2μM PMSF, 1μg/ml aprotinin, and 1μg/ml leupeptin) and lysed in a French pressure cell. The lysate was centrifuged at 30,000g for 1 hour to pellet membrane proteins. The membrane pellet was resuspended in detergent extraction buffer (same as lysis buffer with 4% Triton X-100). Centrifugation was repeated at 30,000g for 30 minutes and the supernatant was filtered serially through 2 micron and 0.8 micron membranes to clarify the lysate. Using a peristaltic pump, the sample was applied to a HiLoad Q anion-exchange column pre-equilibrated with buffer A (50mM Tris pH 8.0, 2mM EGTA/EDTA, 0.1% β-mercaptoethanol, 10% glycerol, 0.1% Triton X-100, 20mM NaCl) on a Pharmacia FPLC system. The column was washed with 1 bed volume of buffer A, then a gradient from 20mM to 1M NaCl was applied over 3 bed volumes. 4mL fractions were collected during the gradient and 20μL was removed from alternating fractions to assay for kinase activity using the phosphocellulose-binding assay. The Neu receptor kinase eluted at approximately 0.4M NaCl. The active fractions were pooled and concentrated using a stirred cell ultrafiltration unit with a YM30 membrane (molecular weight cutoff at 30 kilodaltons). Concentrated Neu was stored in 40% glycerol at -20°C.

To characterize this preparation of Neu, we first showed that Neu is capable of autophosphorylation in the presence of ATP and Mg^{2+} (Fig. 2B). We next determined the K_m for ATP using a continuous spectrophotometric assay (5) with ATP concentrations varying from 20-200 μM. The enzyme obeyed Michaelis-Menten kinetics, and the K_m of Neu receptor for ATP was 32.8 +/- 2.7 μM. A previous study determined the K_m for ATP for a partially purified preparation of Neu to be 26.6 +/- 8.6 μM (6). This preparation of Neu reproducibly resulted in high yields of Neu, and the enzyme was active and suitable for our further experiments.

Task 2. Preparation of peptide libraries. Our synthetic strategy for preparing peptide libraries was described in the June 1998 Progress report. Because we established that residues N-terminal to tyrosine are most important as determinants for Neu phosphorylation, we focused on a library of 20^3 (= 8,000) peptides in which residues both N-terminal to tyrosine have been randomized:

Library I: Ala-Ala-**X**-**X**-**X**-Tyr-Ala-Ala-Arg-Arg-Gly

(where **X** represents an equimolar mixture of all 20 common amino acids at a given position). Analysis of the library by Edman degradation, amino acid analysis, and mass spectrometry indicated that it contained all of the desired sequences in relatively uniform concentrations.

Task 3. Phosphorylation of peptide library by Neu. Neu phosphorylation of peptide library I was carried out using procedures we established previously for PDGF receptor phosphorylation (7). For Neu phosphorylation of peptide library I, the reactions were performed in 50mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 6mM $MnCl_2$, 1mg/ml bovine serum albumin, and 200μM [γ-^{32}P]ATP. 4mM peptide library (total peptide concentration) was allowed to react with Neu in a volume of 1 ml for 1 hour at 30 degrees. After separation of peptides from the reaction mixture using Microcon-10 units, phosphorylated peptides were isolated from non-phosphorylated peptides using a ferric iminodiacetic acid (IDA) column (8). IDA beads were charged with $FeCl_3$, then equilibrated with 12mL of buffer A (50mM MES, 1M NaCl, pH 5.5). The 1mL mixture of peptides and phosphopeptides was diluted ten-fold in buffer A and loaded onto the column. The column was washed with 10mL of buffer A and 10mL of buffer B (2mM MES pH 6.0). The phosphopeptides were eluted with 20mL of 500mM NH_4HCO_3 pH 8.0 and 1mL fractions were collected. Radioactivity was measured by liquid scintillation counting and fractions with radioactivity were pooled. The final volume was reduced to 500μL by SpeedVac. The sample was applied onto a 2.1mm diameter narrowbore HPLC C_{18} column (Applied Biosystems). A 60 minute gradient was run from 0 to 75% acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 200μL/minute. Individual HPLC peaks were collected and counted for radioactivity. One major peak was observed at 28 minutes elution time and one minor peak eluted at 24 minutes (Fig. 3). Radioactivity was confirmed to be present in these peaks by liquid scintillation counting. The peaks were concentrated by SpeedVac to a volume less than 20μL and submitted for sequencing by Edman degradation. Only the major peak contained sufficient peptide for detection. Cycles 3, 4 and 5 corresponding to the degenerate positions of peptide library I yielded Glu, Glu and Ile, respectively. Thus, this experiment resulted in the identification of an optimal substrate sequence for Neu: Ala-Ala-Glu-Glu-Ile-Tyr-Ala-Ala-Arg-Arg-Gly (Table 1).

Task 4. Phosphorylation kinetics of peptide containing optimal sequence. We prepared an individual peptide containing the sequence isolated from Peptide Library I (Ala-Ala-Glu-Glu-Ile-Tyr-Ala-Ala-Arg-Arg-Gly). This peptide was synthesized by standard FMOC procedures, purified by reverse-phase HPLC on a C18 column, and characterized by MALDI-TOF mass spectrometry. Kinase reactions were performed in microtiter plates using a continuous spectrophotometric assay (5). Kinetic parameters (K_m and V_{max}) were calculated by varying peptide substrate concentrations and measuring reaction velocity at 15 second time intervals. Statistical analyses were carried out by fitting initial velocity rates into the Michaelis-Menten equation using MacCurve Fit. Peptide 1 (AAEEIYAARRG) had a V_{max} of 2.4 +/- 0.05 μmol/min/mg and a K_m value of 158.11 +/- 32.2μM (Table 1) These measurements confirm the results of the peptide library study; this peptide is the best sequence reported to date for phosphorylation by Neu.

Objective 2. Studying Neu specificity with a solid-phase kinase assay.

Task 1. Preparing Neu for the solid-phase kinase assay. As described above, Neu was prepared by infecting Sf9 insect cells with baculovirus encoding the V664E Neu (t-Neu) receptor. t-Neu receptor was partially purified by anion chromatography using HiLoad Q Sepharose (Figure 2).

Task 2. Probing solid-phase library with Neu. We modified our strategy for using the solid-phase kinase assay to probe the substrate specificity of Neu. This modification of the procedure allowed us to probe a more highly degenerate library, improving the chances that we would isolate novel substrates for Neu (Figure 4).

Dr. Peter Nestler of Cold Spring Harbor Laboratory has reported that combinatorial libraries may be attached to solid supports for screening (9). Dr. Nestler produced for us a peptide library containing six degenerate positions. This library, Peptide library II, (EDXXXYXXXG, where X represents a degenerate position excluding tyrosine and cysteine) was synthesized using Fmoc chemistry. 10 g of 0.1-0.3mmol/g substituted porous polyethylene beads was used as the starting material. Unreacted free amines on the beads were acetylated using 4 equivalents of acetic anhydride. As with peptide library I, degenerate positions were incorporated according to the "divide-couple-recombine" strategy.

Neu phosphorylation of peptide library II was carried out in 500 μL volumes with 10mg peptide-beads in kinase reaction buffer (100mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 4mM $MnCl_2$, 100μM Na_3VO_4, 1mg/mL bovine serum albumin, and 200μM [γ-^{32}P]ATP). The reaction was incubated at 30 degrees for 6 hours with constant stirring in reaction vials. The peptide-beads were then washed two times in 1mL volumes alternately with 50mM Tris pH 7.4, 1mM ATP and with 50mM Tris pH 7.4, 1mM ATP, 0.5% SDS. This was followed by washing alternately with 0.5% HCl and 0.5% phosphoric acid for a total of ten washes. The peptide-beads were then washed twice with deionized water. Our initial control experiments using peptides with defined sequences confirmed that Neu was capable of phosphorylating immobilized peptides in this form of the solid-phase kinase assay (data not shown). To identify substrate sequences from the library, the large-scale reaction was analyzed as described below under Task 3.

Task 3. Determining the sequences of Neu substrates. In the initial strategy for the solid-phase assay, this task was to be carried out by DNA sequencing of positive clones. This task was modified because the new strategy for identifying positive sequences was based on autoradiography and protein sequencing, as shown in Fig. 4. After reaction with Neu, peptide-beads were then resuspended in 0.5% gelatin (Sigma) and spread evenly on glass plates (9). In a dark room, autoradiography emulsion (Kodak) was spread on the glass plates and allowed to develop for 2 weeks at the temperature of –70°C. Darkly stained beads were extracted using fine forceps and placed in individual glass tubes. The peptide on each bead was identified by Edman degradation. The individual peptides isolated by this solid-phase kinase assay of Neu are presented in Table 1. Most of these sequences contain previously unidentified motifs for phosphorylation by Neu. Interestingly, some of them contain amino acid sequences which bear some resemblance to the sites of autophosphorylation on Neu.

Task 4. Synthesize individual peptides corresponding to optimal sequences; measure kinetics of phosphorylation by Neu. Four individual peptides corresponding to the sequences of phosphorylated peptides from peptide library II were synthesized using Fmoc protocols and used for kinetic analyses. As in Objective 1/Task 4, the peptides were purified by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry to confirm their structures. For the control, a peptide with the sequence AAAAAYAARRG was also prepared and used in the kinetic analyses. Kinase reactions were performed in microtiter plates using the continuous spectrophotometric assay (5). Kinetic parameters (K_m and V_{max}) were calculated by varying peptide substrate concentrations and measuring reaction velocity at 15 second time intervals (Table 1). Statistical analyses were carried out by fitting initial velocity rates into the Michaelis-Menten equation using MacCurve Fit. Accurate kinetic parameters could not be obtained for peptide 2 (EDGPIYQMARRG) by this method because the K_m was too high. The V_{max}/K_m value for peptide 2 was determined graphically using peptide concentrations much lower than K_m. Of the five peptides (i.e., one peptide from Objective 1 and four from Objective 2), peptide 1 and 4 are the best substrates for Neu receptor kinase as seen in their V_{max}/K_m values of 15.1×10^{-3} and

9.6×10^{-3}, respectively. As a control for Neu phosphorylation, a peptide with alanines surrounding the phosphoaccepting tyrosine (AAAAAYAARRG) was used. This peptide had a V_{max} of 1.7 +/- 0.1μmol/min/mg and a K_m value of 1703.5 +/- 245.3μM (Table 1).

Objective 3. Peptide Inhibitors of Neu.

Task 1. Synthesize, purify, and characterize peptide inhibitors of Neu. We attempted to generate Neu inhibitors by replacing the phosphorylatable tyrosine in peptides 1 and 4 (see above). The tyrosine mimetic that we chose for our initial generation of inhibitors was *para*-carboxylphenylalanine (*p*-COOH-F), which has been used successfully by Dr. John McMurray and his collaborators at the M.D. Anderson Cancer Center as a tyrosine mimetic for Src inhibitors (J. McMurray, personal communication). The peptides were synthesized by standard FMOC procedures using a racemic D,L-mixture of FMOC-*p*-COOH-F. The sequences of the inhibitors are given in Table 2. Purification of peptides containing D and L enantiomers of *para*-carboxylphenylalanine (*p*-COOH-F) was carried out using reversed-phased HPLC with an analytical HPLC column. The peptides were purified using a 30 minute gradient from 5 to 75% acetonitrile in 0.1% TFA. For inhibitor 1 (AAEEI-(D,L) *p*-COOH-F-AARRG), absorbance peaks were observed at 21.5 minutes and 22 minutes. For inhibitor 2 (EDKVD-(D,L) *p*-COOH-F-RMHRRG), absorbance was observed at 24.3 minutes. Mass spectral analysis confirmed these peaks to be the correct peptides. To better resolve the D and L diastereomers of inhibitor 1 and to separate the diastereomers of inhibitor 2, we tested an HPLC solvent system consisting of 0-50% acetonitrile in 200mM $NaClO_4$, 25mM NaH_2PO_4, pH 2.5 (10). However, resolution did not improve for inhibitor 1 and separation was not obtained for inhibitor 2.

To identify which peak from the separation of inhibitor 1 corresponded to which diastereomer, the HPLC peaks were analyzed by leucine aminopeptidase digestion (10). Leucine aminopeptidase was preactivated by incubation at 37°C for 1 hour in 100mM Tris pH 8.5, 20mM MnCl2. 100μg of peptide from each peak was added to the preactivated aminopeptidase and digested at 37°C for 3 hours. The digested peptides were desalted and subjected to mass spectral analysis. The first peak (21.5') showed a component with the mass of 387.3, corresponding to residues RRG which has a mass of 388.5. There were no other components with greater mass. The second peak (22') showed a component with the mass of 722, corresponding to residues (D)*p*-COOH-F-AARRG which has a mass of 721.8. As leucine aminopeptidase recognizes only L-amino acids, it was concluded that the first peak corresponded to the peptide containing the L enantiomer of *p*-COOH-F (inhibitor L-1) and the second peak contained the D enantiomer.

Task 2. Test peptides as inhibitors of Neu in vitro. Peptide-based inhibitors were synthesized using *para*-carboxylphenylalanine as the replacement for tyrosine in peptides 1 and 4. The D and L diastereomers of inhibitor 1 were separated as described above; the two diastereomers of peptide inhibitor 2 could not be resolved. The inhibition constant K_i was determined for the two diastereomers of peptide inhibitor 1 and for the diastereomeric mixture of peptide inhibitor 2. K_i was measured using the continuous spectrophotometric assay (5), with varying amounts of inhibitor (50-2000μM) and two fixed concentrations of peptide substrate (300 and 600μM). K_i values were determined graphically according to the method of Dixon (11). The values are presented in Table 2. Thus, the best inhibitor of Neu in this set is the L enantiomer of inhibitor 1, with a K_i of 1.6 mM.

The relatively weak inhibitory potency of these peptides suggests to us that they would perform poorly in the studies proposed for Technical Objective 4 (in vivo inhibition). Thus, we will first prepare additional peptides based on peptide 1 in which the phosphorylatable tyrosine is replaced with other tyrosine mimetics. Among the tyrosine surrogates that we will consider incorporating are: Phenylalanine (12), Tetrafluorotyrosine (13), and L- DOPA (14). We will follow the strategy described above to produce and test these peptides in vitro using purified full-length Neu. After these experiments, we will select the best inhibitor and test it on other tyrosine-

and serine/threonine-protein kinases (Objective 3/Task3), then proceed to the in vivo experiments in Objective 4.

Task 3: Analyze pattern of inhibition for most potent inhibitors; test inhibitors against other protein kinases. Not yet addressed.

Objective 4. In vivo inhibition of Neu. Not yet addressed.

FIGURE LEGENDS

Figure 1. Expression of the catalytic domain of Neu as a fusion protein with glutathione S-transferase (GST) in bacteria. Lane 1: crude cell lysate of E. coli cells expressing GST-Neu; Lanes 2 and 3, 10 µl and 5 µl of purified GST-Neu, respectively.

Figure 2. A) Full-length t-Neu was expressed in Sf9 cells. Lanes 1 and 2 show two separate infections of Sf9 cells with baculovirus encoding t-Neu (transforming Neu with V664E mutation). Lanes 3 and 4 show infections using the cytoplasmic domain of Neu alone. Cells were harvested and lysates were electrophoresed in 8% SDS-PAGE. Protein was transferred overnight onto PVDF membrane and probed with monoclonal anti-phosphotyrosine antibody 4G10 (Upstate). B) t-Neu was partially purified and used in an autophosphorylation assay. Lanes 1 and 2, two separate infections of Sf9 cells with Neu; crude cell lysates are shown. Lane 3, partially purified Neu; Lane 4, autophosphorylated Neu. The reaction was electrophoresed in 8% SDS-PAGE, transferred onto PVDF membrane and probed with monoclonal anti-phosphotyrosine antibody 4G10.

Figure 3. Identification of Neu-Phosphorylated Peptide from Peptide Library I. HPLC analysis was carried out on a narrowbore C18 column, as described in the text. The major peak was determined by Edman sequencing to contain Peptide 1 (AAEEIYAARRG). The minor peak did not contain any peptides in sufficient quantity for Edman sequencing.

Figure 4. Strategy for isolating Neu substrate peptides using a solid-phase kinase assay.

Table 1. Substrate sequences for Neu identified in this study, along with kinetic parameters for phosphorylation.

Table 2. Sequences of peptide inhibitors and inhibitory constants.

Figure 1. Expression and purification of GST-Neu.

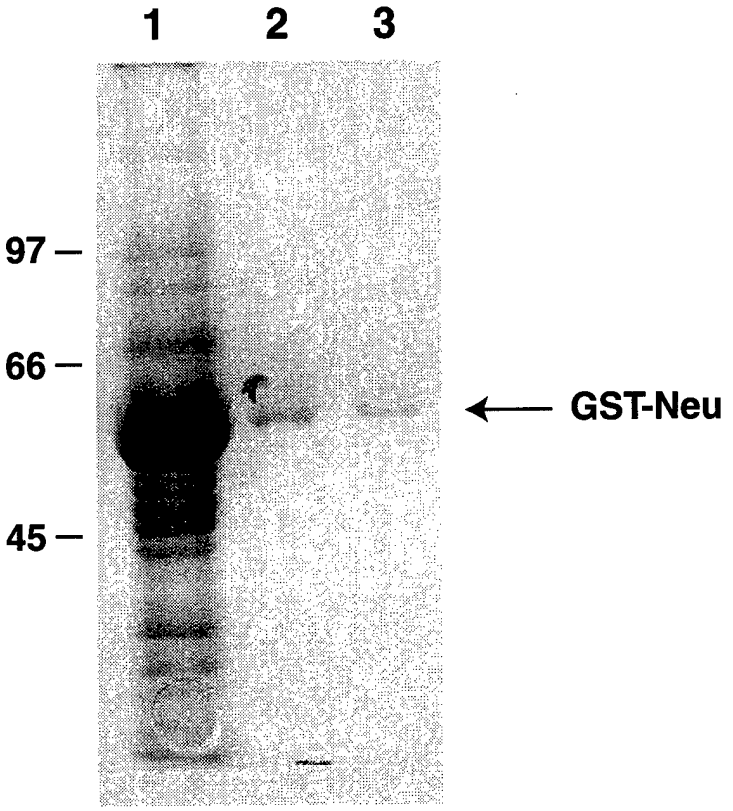

Figure 2. Expression and purification of Neu.

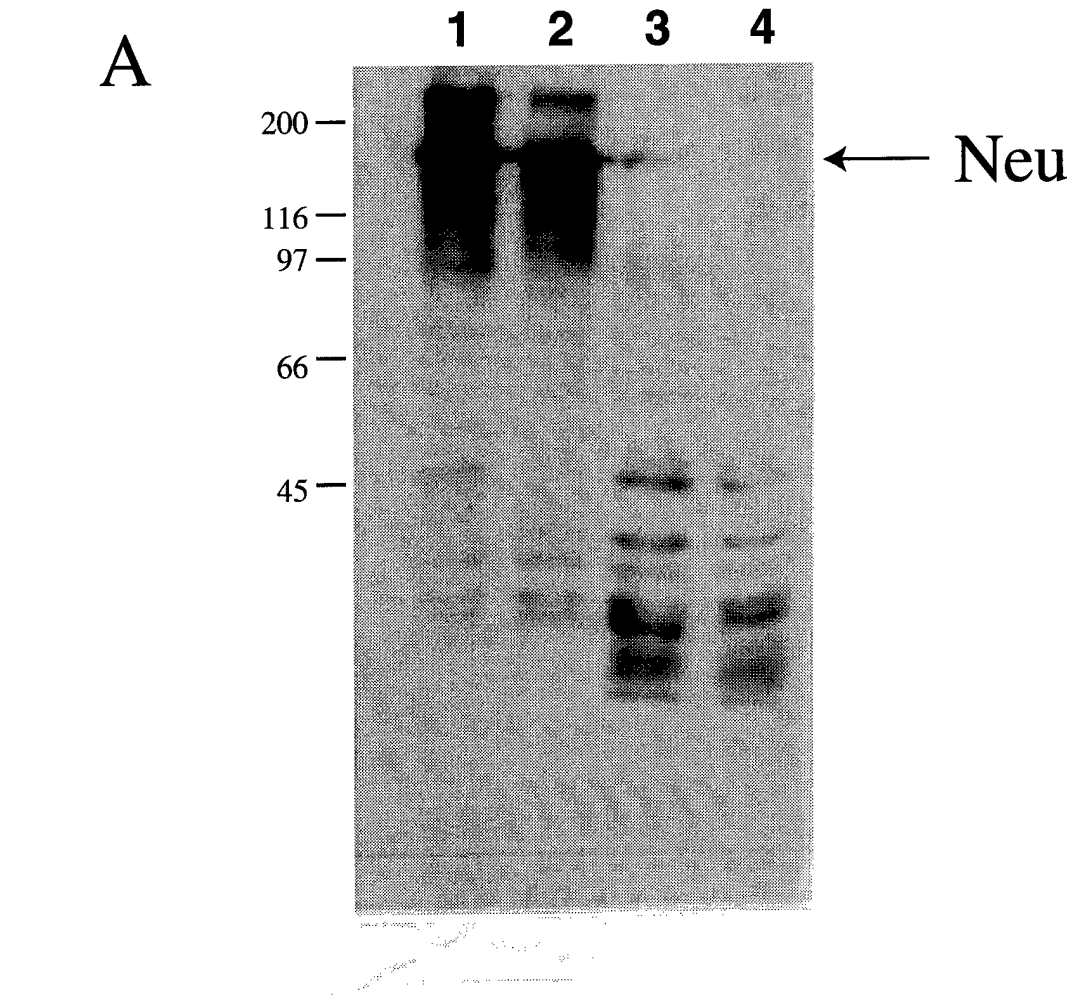

Figure 3. HPLC Analysis of Neu-Phosphorylated Peptides

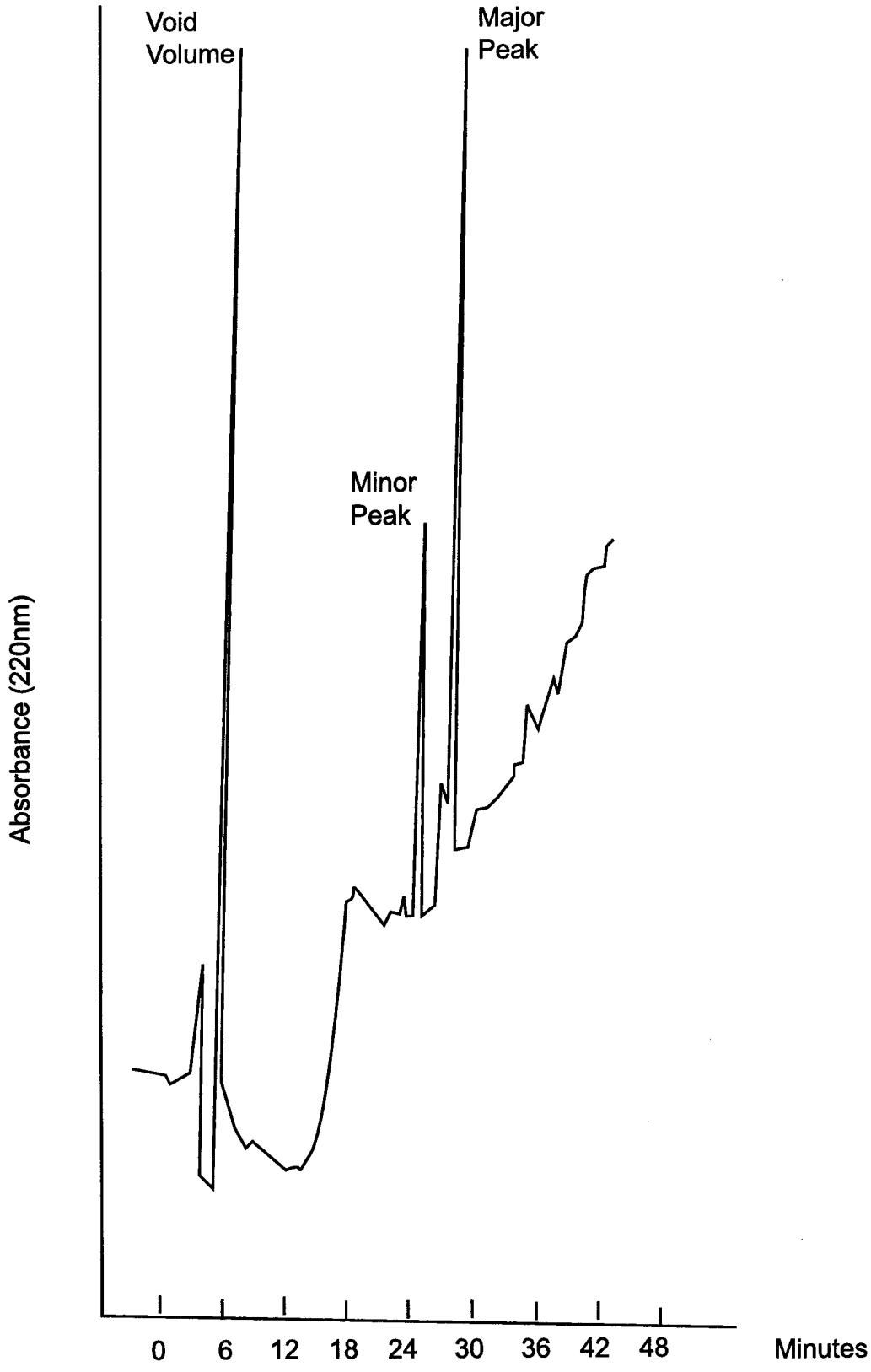

Figure 4. Solid-Phase Kinase Assay

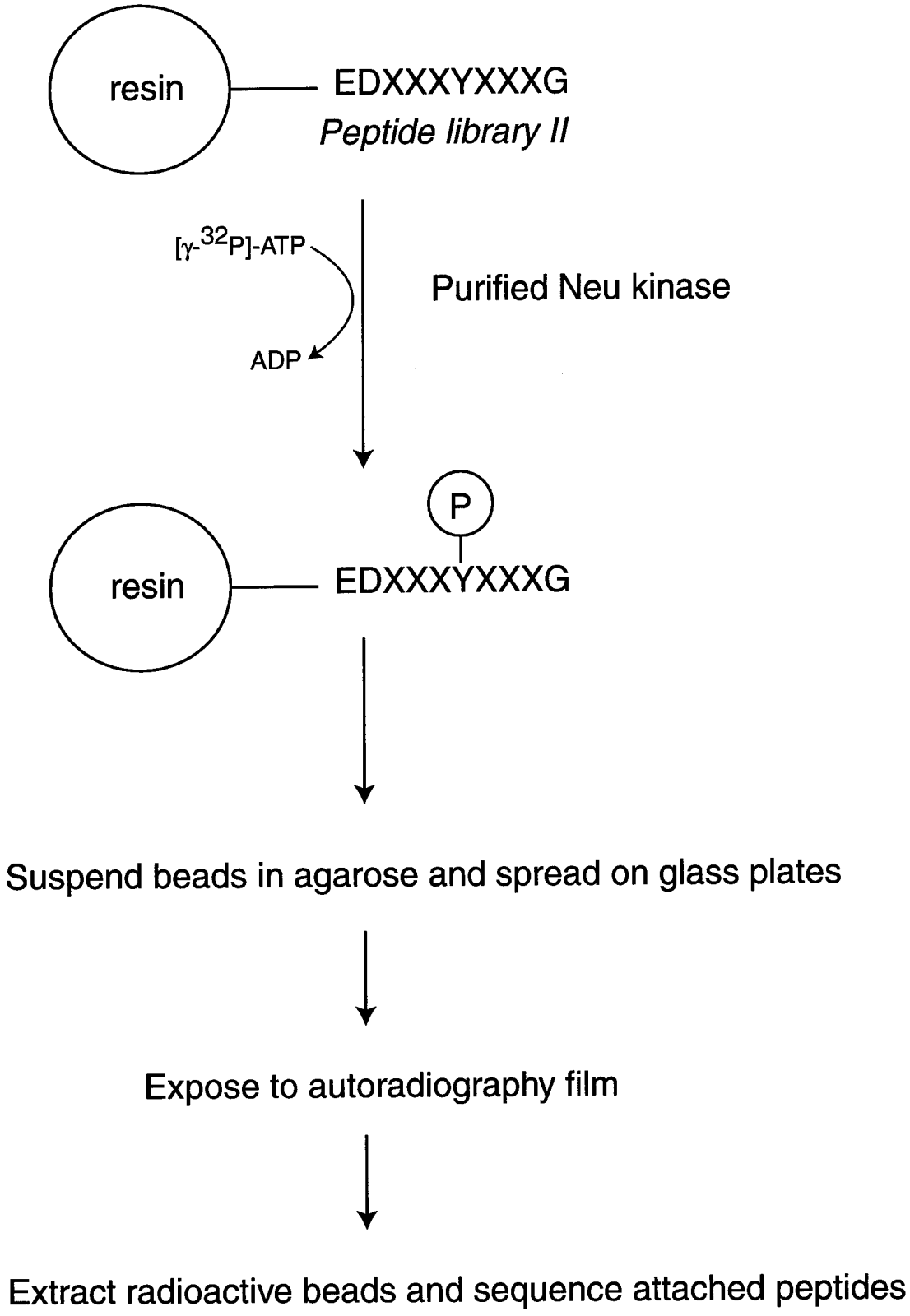

Table 1. Neu substrate sequences identified from Peptide libraries I and II, and kinetic analyses of individual peptides

Peptide Sequence	Library used	V_{max} (μmol/min/mg)	K_m (μM)	V_{max}/K_m (10^{-3})
1. AAEEIYAARRG	I	2.4 +/- 0.05	158.11 +/- 32.2	15.1
2. EDGPIYQMARRG	II	n.d.	n.d.	0.02*
3. EDFAMYLNSRRG	II	2.6 +/- 0.02	382.6 +/- 38.1	6.8
4. EDKVDYRMHRRG	II	2.35 +/- 0.23	245.2 +/- 28.7	9.6
5. EDFQKYKMLRRG	II	5.9 +/- 0.07	1401.3 +/- 233.1	4.2
6. AAAAAYAARRG	control	1.7 +/- 0.1	1703.5 +/- 245.3	1.0

Sequences isolated from Peptide library I (Technical Objective 1) and Peptide library II (using the assay described in Technical Objective 2). Underlined residues correspond to the degenerate positions of the two peptide libraries. Peptides corresponding to the sequences identified from the screening of peptide libraries I and II were synthesized. Kinetic analyses of Neu receptor kinase were carried out using the continuous spectrophotometric assay (5). Peptide 6 contains a control sequence. *Phosphorylation rates for peptide 2 were too low to measure kinetic parameters using this assay. The V_{max}/K_m value was determined graphically by using substrate concentrations that were much lower than K_m.

Table 2. K_i values of Peptide-based Inhibitors.

Peptide Inhibitor	K_i (mM)
D-1. AAEEI(D- *p*-COOH-F)AARRG	4.29
L-1. AAEEI(L- *p*-COOH-F)AARRG	1.61
2. EDKVD(D,L- *p*-COOH-F)RMHRRG	7.77

Table 2. K_i values for the peptide-based inhibitors were determined graphically from the Dixon plots (1/V vs. [I]).

KEY RESEARCH ACCOMPLISHMENTS.

- Produced full-length, active form of Neu
- Tested substrate specificity of Neu using two combinatorial library techniques
- Identified five novel substrate sequences for Neu. Confirmed using individual peptides.
- Synthesized and tested first-generation peptide inhibitors of Neu.

REPORTABLE OUTCOMES.

1. A manuscript describing the results obtained in Technical Objectives 1 and 2 is in preparation.

2. The results described above will form a major part of the Ph.D. thesis of Perry M. Chan, a student in the Program in Molecular and Cellular Biology at SUNY Stony Brook.

CONCLUSIONS

The overall goal of this project is to characterize the substrate specificity of the Neu tyrosine kinase. In the second year of this project, we have isolated the Neu protein from Sf9 insect cells using a baculovirus expression vector. The Neu that we have prepared from these sources is active. We report here the results of experiments conducted using combinatorial peptide libraries, including a solid-phase kinase assay. We have isolated five novel sequences from the libraries that are phosphorylated efficiently by Neu: AAEEIYAARRG, EDGPIYQMARRG, EDFAMYLNSRRG, EDKVDYRMHRRG, and EDFQKYKMLRRG, where the underlined sequence represents the randomized positions. We confirmed that these sequences represent Neu substrates using individually-synthesized peptides, and determined the kinetic parameters for phosphorylation by Neu. One of them (AAEEIYAARRG) is the best synthetic peptide substrate reported to date for Neu. We synthesized potential peptide-based inhibitors of Neu by replacing the phosphorylatable tyrosine in this peptide and another peptide with para-carboxy-Phe. The inhibitory potency of this first generation of inhibitors toward purified Neu is somewhat low. Thus, we plan to prepare and test additional inhibitors containing other tyrosine mimetics. We will test the best inhibitor on other tyrosine- and serine/threonine-protein kinases in vitro, then test it as an in vivo inhibitor of Neu.

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